12-13-01

JC10 Rec'd PCT/PTO 29 OCT 2001

Form PTO-1390 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE
(REV 11-2000)

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
PL-9915	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	
To be assigned	10/018021

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/04104	May 8, 2000	May 17, 1999

TITLE OF INVENTION
Method for the Purification of Protein Kinase by Affinity Chromatography

APPLICANT(S) FOR DO/EO/US
Mart Loog, Asko Uri, Jaak Jarv, and Pia Ek

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

duplicate copy of this transmittal letter for charging purposes
return postcard

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
To be assigned 10/018021	PCT/EP00/04104	PL-9915

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1040.00**	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$890.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$740.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$710.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**	

ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =	0	x $18.00	**$0.00**	
Independent claims	1 - 3 =	0	x $84.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	

TOTAL OF ABOVE CALCULATIONS =	**$890.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	**$0.00**	
SUBTOTAL =	**$890.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	**$0.00**	
TOTAL NATIONAL FEE =	**$890.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	**$0.00**	
TOTAL FEES ENCLOSED =	**$890.00**	
	Amount to be: refunded	$
	charged	$

a. ☐ A check in the amount of _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **500-588** in the amount of **$890.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **500-588** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Royal N. Ronning, Jr.
Amersham Biosciences
800 Centennial Avenue
Piscataway, New Jersey 08855

(732) 457-8423

SIGNATURE

Royal N. Ronning, Jr.
NAME

32,529
REGISTRATION NUMBER

October 29, 2001
DATE

PL-9915

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	M. Loog, et al.	Group Art Unit:	To be assigned
Serial Number:	To be assigned	Examiner:	To be assigned
Filing Date:	October 29, 2001		
Title:	Method for the Purification of Protein Kinase by Affinity Chromatography		

FIRST PRELIMINARY AMENDMENT

Honorable Assistant Commissioner of Patents
Box Patent Application
Washington, D.C. 20231

Sir:

Please consider the following amendments and remarks in connection with the prosecution of the captioned application, which is a filing under 35 U.S.C. § 371 and claims priority to international application number PCT/EP00/04104 filed May 8, 2000. This application also claims priority to Swedish patent application number 9901807-9 filed May 17, 1999.

In the Claims

Please amend page 20, line 1, as follows:

[C L A I M S]

What is claimed is:

Please amend claim 1 as follows:

1. (once amended) A method for removal of protein kinase from a liquid containing the protein kinase [by]comprising contacting the liquid with a carrier bound affinity ligand for the kinase, [**characterized** in that the]wherein said ligand is a bifunctional inhibitor for the kinase.

Please amend claim 2 as follows:

2. (once amended) The method of claim 1, [**characterized** in that]wherein the bifunctional inhibitor comprises the structure

$$C - (L)_n - N$$

where

(a) C contains a structure inhibiting binding of the peptide/protein substrate to the protein kinase,

(b) L is an organic linker,

(c) n is an integer 0 or 1, and

(d) N is aninhibitor competitively inhibiting binding of the nucleoside triphosphate (NTP) to the protein kinase.

Please amend claim 3 as follows:

3. (once amended) The method of [any one of claims 1-2]claim 2, [**characterized** in that]wherein C is a peptide substrate consensus sequence or a pseudosubstrate consensus sequence.

Please amend claim 4 as follows:

4. (once amended) The method of claim 3, [**characterized** in that]wherein C comprises at least one basic amino acid residue[, preferably selected from arginine, lysine and hydroxylysine, when] and the protein kinase has basic substrate specificity determinants.

Please amend claim 5 as follows:

5. (once amended) The method of claim 3, [**characterized** in that]wherein C comprises one acidic amino acids[, preferably selected from aspartic acid and glutamic acid, when] and the protein kinase has acidic substrate specificity determinants.

Please amend claim 6 as follows:

6. (once amended) The method of [any one of claims 3-5]claim 3, [**characterized** in that]wherein C comprises at least one hydrophobic amino acid residue[, preferably selected among leucine, isoleucine, phenylalanine].

Please amend claim 7 as follows:

7. (once amended) The method of [any one of claims 3-6]claim 3, [**characterized** in that]wherein C comprises at least two [amino acid]acidic amino acids and/or at least two basic amino acids[, with preference for these amino acid residues being in sequence].

Please amend claim 8 as follows:

8. (once amended) The method of [any one of claims 3-7]claim 3, [**characterized** in that]wherein C comprises at least two arginine residues and/or at least two aspartic acid residues.

Please amend claim 9 as follows:

9. (once amended) The method of [any one of claims 2-8]claim 2, [**characterized** in that]wherein N is a structure comprising a nucleotide structure[, preferably adenosine or guanosine with] and the protein kinase [being]is capable of [phosphorylate]phosphorylating with ATP and GTP, respectively.

Please amend claim 10 as follows:

10. (once amended) The method of claim 9, [**characterized** in that]wherein the nucleotide structure is covalently bound to L utilizing its 5' –carbon.

Please amend claim 11 as follows:

11. (once amended) The method of claim 10, [**characterized** in that]wherein the 5' – carbon is in the form of a derivatized carboxylic acid.

Please amend claim 12 as follows:

12. (once amended) The method of [any one of claims 2-11]claim 2, [**characterized** in that]wherein L is a peptide chain.

Please amend claim 13 as follows:

13. (once amended) The method of [anyone of claims 2-12]claim 2, [**characterized** in that]wherein L is a peptide chain composed of non-α- and/or non-L amino acids.

Please amend claim 14 as follows:

14. (once amended) The method of [any one of claims 1-15]claim 1, [**characterized** in that]wherein the carrier is insoluble in the liquid.

Please add new claim 15 as follows:

15. (new) The method of claim 4 wherein the basic amino acid residue is selected from the group consisting of arginine, lysine, and hydroxylysine.

Please add new claim 16 as follows:

16. (new) The method of claim 5 wherein the acidic amino acid residue is selected from the group consisting of aspartic acid and glutamic acid.

Please add new claim 17 as follows:

17. (new) The method of claim 6 wherein the hydrophobic amino acid residue is selected from the group consisting of leucine, isoleucine, and phenylalanine.

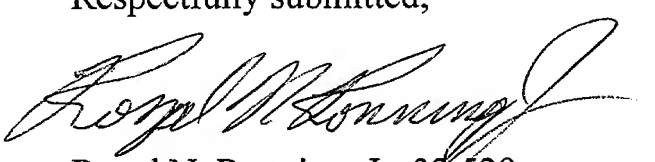
Remarks

Claims 1-14 are pending in the instant application. Applicants have amended claims 1-14 to more fully conform with U.S. practice and to delete multiple dependencies. Applicants have also added new claims 15-17. A version of the claims marked up to show the amendments, as well as a clean version of the claims encompassing the amendments, is attached hereto.

Applicants respectfully assert that all amendments are fairly based on the specification, and respectfully request their entry.

Applicants believe that the claims, as amended, are in allowable form, and earnestly solicit the allowance of claims 1-17.

Respectfully submitted,



Royal N. Ronning, Jr. 32,529
Attorney for Applicants

Amersham Biosciences
800 Centennial Avenue
P. O. Box 1327
Piscataway, New Jersey 08855-1327

Tel: (732) 457-8423
Fax: (732) 457-8463

H:\DEPT\Law_Dept\IP Dept\Amendments\First Preliminary Amendment-PL9915 (10-29-01).doc

Claims (marked-up version showing amendment(s))

Page 20, line 1:

[C L A I M S]

What is claimed is:

1. (once amended) A method for removal of protein kinase from a liquid containing the protein kinase [by]comprising contacting the liquid with a carrier bound affinity ligand for the kinase, [**characterized** in that the]wherein said ligand is a bifunctional inhibitor for the kinase.

2. (once amended) The method of claim 1, [**characterized** in that]wherein the bifunctional inhibitor comprises the structure

$$C-(L)_n-N$$

where

(a) C contains a structure inhibiting binding of the peptide/protein substrate to the protein kinase,

(b) L is an organic linker,

(c) n is an integer 0 or 1, and

(d) N is aninhibitor competitively inhibiting binding of the nucleoside triphosphate (NTP) to the protein kinase.

3. (once amended) The method of [any one of claims 1-2]claim 2, [**characterized** in that]wherein C is a peptide substrate consensus sequence or a pseudosubstrate consensus sequence.

4. (once amended) The method of claim 3, [**characterized** in that]wherein C comprises at least one basic amino acid residue[, preferably selected from arginine, lysine and hydroxylysine, when] and the protein kinase has basic substrate specificity determinants.

5. (once amended) The method of claim 3, [**characterized** in that]wherein C comprises one acidic amino acids[, preferably selected from aspartic acid and glutamic acid, when] and the protein kinase has acidic substrate specificity determinants.

6. (once amended) The method of [any one of claims 3-5]claim 3, [**characterized** in that]wherein C comprises at least one hydrophobic amino acid residue[, preferably selected among leucine, isoleucine, phenylalanine].

7. (once amended) The method of [any one of claims 3-6]claim 3, [**characterized** in that]wherein C comprises at least two [amino acid]acidic amino acids and/or at least two basic amino acids[, with preference for these amino acid residues being in sequence].

8. (once amended) The method of [any one of claims 3-7]claim 3, [**characterized** in that]wherein C comprises at least two arginine residues and/or at least two aspartic acid residues.

9. (once amended) The method of [any one of claims 2-8]claim 2, [**characterized** in that]wherein N is a structure comprising a nucleotide structure[, preferably adenosine or guanosine with] and the protein kinase [being]is capable of [phosphorylate]phosphorylating with ATP and GTP, respectively.

10. (once amended) The method of claim 9, [**characterized** in that]wherein the nucleotide structure is covalently bound to L utilizing its 5' –carbon.

11. (once amended) The method of claim 10, [**characterized** in that]wherein the 5' –carbon is in the form of a derivatized carboxylic acid.

12. (once amended) The method of [any one of claims 2-11]claim 2, [**characterized** in that]wherein L is a peptide chain.

13. (once amended) The method of [anyone of claims 2-12]claim 2, [**characterized** in that]wherein L is a peptide chain composed of non-α- and/or non-L amino acids.

14. (once amended) The method of [any one of claims 1-15]claim 1, [**characterized** in that]wherein the carrier is insoluble in the liquid.

15. (new) The method of claim 4 wherein the basic amino acid residue is selected from the group consisting of arginine, lysine, and hydroxylysine.

16. (new) The method of claim 5 wherein the acidic amino acid residue is selected from the group consisting of aspartic acid and glutamic acid.

17. (new) The method of claim 6 wherein the hydrophobic amino acid residue is selected from the group consisting of leucine, isoleucine, and phenylalanine.

Claims (clean version encompassing amendments)

What is claimed is:

1. (once amended) A method for removal of protein kinase from a liquid containing the protein kinase comprising contacting the liquid with a carrier bound affinity ligand for the kinase, wherein said ligand is a bifunctional inhibitor for the kinase.

2. (once amended) The method of claim 1, wherein the bifunctional inhibitor comprises the structure

$$C - (L)_n - N$$

where

(a) C contains a structure inhibiting binding of the peptide/protein substrate to the protein kinase,

(b) L is an organic linker,

(c) n is an integer 0 or 1, and

(d) N is aninhibitor competitively inhibiting binding of the nucleoside triphosphate (NTP) to the protein kinase.

3. (once amended) The method of claim 2, wherein C is a peptide substrate consensus sequence or a pseudosubstrate consensus sequence.

4. (once amended) The method of claim 3, wherein C comprises at least one basic amino acid residue and the protein kinase has basic substrate specificity determinants.

5. (once amended) The method of claim 3, wherein C comprises one acidic amino acids and the protein kinase has acidic substrate specificity determinants.

6. (once amended) The method of claim 3, wherein C comprises at least one hydrophobic amino acid residue.

7. (once amended) The method of claim 3, wherein C comprises at least two acidic amino acids and/or at least two basic amino acids.

8. (once amended) The method of claim 3, wherein C comprises at least two arginine residues and/or at least two aspartic acid residues.

9. (once amended) The method of claim 2, wherein N is a structure comprising a nucleotide structure and the protein kinase is capable of phosphorylating with ATP and GTP, respectively.

10. (once amended) The method of claim 9, wherein the nucleotide structure is covalently bound to L utilizing its 5' –carbon.

11. (once amended) The method of claim 10, wherein the 5' –carbon is in the form of a derivatized carboxylic acid.

12. (once amended) The method of claim 2, wherein L is a peptide chain.

13. (once amended) The method of claim 2, wherein L is a peptide chain composed of non-α- and/or non-L amino acids.

14. (once amended) The method of claim 1, wherein the carrier is insoluble in the liquid.

15. (new) The method of claim 4 wherein the basic amino acid residue is selected from the group consisting of arginine, lysine, and hydroxylysine.

16. (new) The method of claim 5 wherein the acidic amino acid residue is selected from the group consisting of aspartic acid and glutamic acid.

17. (new) The method of claim 6 wherein the hydrophobic amino acid residue is selected from the group consisting of leucine, isoleucine, and phenylalanine.

METHOD FOR THE PURIFICATION OF PROTEIN KINASE BY AFFINITY CHROMATOGRAPHY

Technical field

The invention concerns a method for the removal of a protein kinase from a liquid by contacting the liquid with a carrier carrying ligands having affinity for the kinase concerned. The removal may be a part step in the purification of the protein kinase.

The terms "protein" and "protein" are used interchangeable if not said otherwise.

Background technology - purification of protein kinases by chromatography

Protein kinases have previously been purified by a combination of chromatographic procedures, such as ion exchange and/or hydrophobic interaction chromatography (IEC and HIC, respectively) (Ogita K et al., Methods Enzymol. 200 (1991) 228-34; and Huang KP et al., Methods Enzymol. 200 (1991) 241-52). Other chromatographic media used have been based on immobilized affinity ligands, such as

- Adenosine triphosphate (Haystead CM et al., Eur. J. Biochem. 214(2) (June 1993) 459-67; and Jeno P et al., Methods Enzymol 200 (1991) 178-87),
- Polyionic inhibitor (heparin) (Khilko S et al., Protein Expr. Purif. 3(5) (Oct 1992) 355-61),
- Peptide inhibitor (Olsen SR et al., J. Biol. Chem. 264(31) (Nov 1989) 18662-6; and
- Inhibitor competing with ATP (Swanson KD et al., J Biol Chem 274(6) (Feb 1999) 3385-95).

Background technology - protein kinases, substrate and inhibitors

Protein kinases act by bringing the appropriate nucleotide triphosphate (NTP) and protein substrate together to form an

NTP protein/peptide bisubstrate complex thereby enabling transfer of a phosphate group from NTP to a phosphate accepting amino acid of the protein substrate. The substrate binding area on the protein kinase has one NTP-binding pocket and one protein substrate binding groove. For most protein kinases NTP is adenosine triphosphate (ATP) with a few known exceptions that utilize guanosine triphosphate (GTP).

The minimal specific amino acid primary structure motif (sequence) around a phosphate accepting amino acid necessary for efficient phosphorylation by a kinase is called the substrate consensus motif (sequence) for the kinase. A number of substrate consensus motifs are known. See for instance Pearson et al., Methods Enzymol. 200 (1991) 62-.

At least three different kinds of protein kinase inhibitors are previously known.

1. A first kind of inhibitor relates to compounds competing with the relevant NTP (for instance ATP) for binding to the NTP-binding pocket on a kinase or close thereto thereby blocking binding of the NTP relevant to the protein kinase. This kind of inhibitor comprises compounds that have one or more ring systems that may be condensed or not condensed, heterocyclic or not heterocyclic, aromatic or not aromatic etc. Various derivatives of nucleotides having no free triphosphate group are potential inhibitors of this kind. Isoquinoline sulphonamide and its analogues (Hidaka et al., Biochemistry 23 (1984) 5036-5041), and staurosporine and its analogues (Takahashi et al., J. Pharmacol. Exp. Ther. 255 (1990) 1218-1221; Meyer et al., Int. J. Cancer 43 (1989) 851-856; and Davis et al., FEBS Letters 259(1) 1989) 61-63) are other illustrative examples. Due to the highly conserved structure in the NTP binding pocket, this kind of inhibitors will have a poor selectivity with respect to different protein kinases.
2. A second kind of inhibitor binds to the peptide groove or close thereto thereby blocking binding of the normal

protein/peptide substrate. This kind of inhibitor encompasses substrate consensus motifs, e.g. containing a pseudosubstrate peptide, and inhibits the binding of the native protein substrate to the protein kinase concerned (Walsh et al., Methods Enzymol. 201 (1991) 304-316; and Kemp et al., Methods Enzymol. 201 (1991) 287-304) as well as non-peptide structures competing with the protein/peptide substrate binding site..

3. A third kind of inhibitors are bifunctional. The inhibitors of this kind are covalent conjugates between one moiety inhibiting NTP binding and one moiety inhibiting binding of a protein/peptide substrate to the protein kinase (Ricourt et al., J. Med. Chem. 34 (1991) 73-; Medzihradszky et al., J. Am. Chem. Soc. 116 (1994) 9413-). A study on the optimisation of the linker structure between the moiety mimicking NTP and the moiety mimicking the protein substrate has been published recently (Loog et al., Cell. Mol. Biol. Let. 3(39 (1998) 317, Poster at The first International Conference on Inhibitors of Protein Kinases, September 15-20, 1989, Warsaw, Poland).

Conjugates between adenosine-5'-carboxylic acids and amino acids and oligopeptides containing basic or acidic amino acids have been described previously. These conjugates have been studied as potential ligands for P_2-purinergic receptors (Uri et al., Bioorg. Med. Chem. 2(10) (1994) 1099-1105; and Pehk et al., Bioorg. Med. Chem. Lett. 7(17) (1997) 2159-2164).

The objects of the invention.

- A first object is to provide an improved method for the removal of a protein kinase from a liquid, including purification, by the use of a carrier having an immobilized affinity ligand for a protein kinase.
- A second object is to provide immobilized affinity ligands that promote removal of a protein kinase from a liquid with an improved specificity.

- A third object is to provide immobilized protein kinase affinity ligands that are able to discriminate between various kinds or groups of protein kinases, i.e. improved selective protein kinase binding ligands.

The term "immobilized" means that the affinity ligand is immobilized to a carrier that may or may not be soluble in aqueous liquid media, such as water solutions. Immobilization may take place either before or after binding to the protein kinase.

The invention

It has now been recognised that these objects can be met for a predetermined protein kinase, if the affinity ligand is selected from bifunctional inhibitor analogues, i.e. inhibitors that have a structure containing:

(a) a moiety (N) that has a structure that is able to competitively inhibit binding of the relevant NTP to the protein kinase, and

(b) a moiety (C) that has a structure that is able to competitively inhibit binding of a peptide/protein substrate to the protein kinase.

The method of the invention encompasses that an aqueous liquid containing a protein kinase that is to be removed is contacted with a carrier to which a ligand is attached. The conditions are selected such that the protein kinase becomes bound (adsorbed) to the carrier via the ligand. In the preferred embodiments a plurality of the same or different protein kinase binding ligands is attached to a base matrix or is possible to attach to a base matrix after the ligands have become bound to the protein kinase. The characteristic feature of the method is that the ligand is a bifunctional inhibitor analogue for the protein kinase.

The ligand

A bifunctional inhibitor analogue may be regarded as a chemical conjugate between the N-moiety and C-moiety defined above:

$$C-(L)_n-N$$

where L is an organic linker n is an integer 0 or 1. When n = = 0, a functional group in a compound comprising the structure of moiety C and a functional group in a compound comprising the structure of moiety N has been linked together without creation of any extra linker chain.

The ligand (= bifunctional inhibitor analogue) in turn is attached to the matrix, possibly via a spacer (see below under heading "The matrix and the attachment of the ligand thereto"):

$$[C-(L)_n-N]\text{-------}\ M$$

where

- M represents the matrix and includes any spacer that may be present;
- ------- represents that the matrix binds to the bifunctional inhibitor analogue in the C or N moiety or in the L moiety.

M may contain additional ligand groups binding to a protein kinase as defined herein. Such additional groups may have different or identical structure as the one referred to in the formula above.

Moiety C is a non-phosphorytable structure that is capable of competitively inhibiting binding of a peptide/protein substrate to a protein kinase. Moiety C thus binds either at the protein substrate binding site (peptide binding groove) or close thereto thereby blocking the site.

Moiety C may be a substrate consensus motif (sequence) in which the phosphorylatable amino acid is missing or is blocked from becoming phosphorylated or is replaced with a non-phosphorylated amino acid. Such structure can be also called a

pseudosubstrate. Typical substrate consensus sequences comprise a phosphorytable amino acid (phosphate accepting amino acid) and, within a distance of ± 15 amino acids, one or more preselected amino acids that make the sequence recognisable by one or more selected protein kinases. Many times the preselected amino amino acids are localized within a shorter distance from the phosphorylatable amino acid, for instance within a distance of ± 12 or within ± 10 amino acids. The preselected amino acids may be basic, acidic, hydrophobic, contain amide groups, proline etc. The specified amino acids may be 1, 2 or more amino acids of the same kind that may or may not be arranged in sequence.

Moiety C may also be a peptide sequence containing a number of juxta-positioned acidic amino acids or basic amino acids. The number of such juxta-positioned amino acids may be 2-30 or even higher.

In moiety C of the above-mentioned kinds there may also be one or more hydrophobic amino acid in the carboxy or amino direction of the amino acid sequence (from the position of the phosphorytable amino acid). The hydrophobic amino acid(s) is often located within a distance of ± 15 amino acids atoms from the phosphorylatable amino acid or from a position where it should have been. Depending on protein kinase looked at, the distance may be 1, 2, 3 or 4 or more amino acids.

When moiety C is an amino acid sequence, C may comprise 2-50 or even more amino acids.

Moiety C may also be a none-peptide structure that competitively inhibits the binding of a normal protein substrate to its protein kinase. Illustrative examples are heparin and peptidomimetics.

Arginine, lysine and hydroxylysine are examples of basic amino acids. These amino acids are preferably present in

moiety C when C is an amino acid sequence having affinity for protein kinases with basic substrate specificity determinants.

Aspartic acid and glutamic acid are examples of acidic amino acids. They are preferably present in moiety C when it is an amino acid sequence having affinity for protein kinases with acidic substrate specificity determinants.

Glutamine and asparagine are amino acids that have an amide group.

Serine, threonine, histidine, tyrosine, hydroxyproline are examples of phosphorylatable amino acids.

Leucine, isoleucine, valine, and phenylalanine are examples of hydrophobic amino acids.

Moiety N is selected from compounds comprising the nucleotide structure concerned, for instance the corresponding nucleotide or a compound having a structure capable of competitively inhibiting binding of NTP to a protein kinase. This means that N is capable of binding to the NTP-pocket or close thereto thereby blocking the pocket (NTP active site).

- For NTP being adenosine triphosphate this is best illustrated by the first kind of inhibitors discussed above.

For NTP being guanine triphosphate, compounds analogously related to guanine may be selected as N.

Compounds suitable to be used as N and C may be tested in inhibition experiments as outlined in the experimental part.

The organic linker (L) may vary between different protein kinases and is likely to also depend on the selection of C and N. L should be selected among groups providing a chain comprising from 1-50 atoms, for instance 1-30 atoms, such as 1-18 atoms. In addition to the chain as such there may be groups projecting from the chain. The chain may comprise one or more groups selected from amides (-CONR'-, where R', for

instance, is selected from the side groups present in amino acids), amines (-NR''-, where R'', for instance, is selected from lower alkyl (C_{1-5} alkyl)), azo (-N=N-), ether (-O-), thioether (-S-), bivalent hydrocarbon groups etc.

The chain may be built up of amino acid residues of the α-, β-, δ-, γ- or ε-type or of the L or the D-type or mixtures of these. The chain may carry one or more positive and/or negative groups, for instance primary, secondary, tertiary and quartenary ammonium groups respective carboxy and sulphonate groups. In case L is a peptide chain it may contain from 1-15 amino acid residues or even more.

One type of linkers comprises a hydrocarbon chain which

(a) is straight branched or cyclic and possibly is interrupted at one or more positions by an oxygen or a nitrogen and/or

(b) has one or more carbons that are substituted with an $-NR_1R_2$ or an $-OR_3$ group in which R_{1-3} is selected amongst C_{1-10} alkyl.

One kind of linkers complying with this has the formula $-((CH_2)_{n'''}O-)_m$ where n''' is an integer 1, 2, 3 or 4 and m may be an integer 1-18.

L may be attached to the C and N moieties at various locations. When N is a nucleotide structure, the attachment of L to N should be at the carbohydrate part of N. When C is an amino acid sequence, the linker L should be attached to C at either the carboxy or the amino terminal in C, with the latter being the preferred location. An additional alternative is to attach L at a side group in an amino acid present in C, for instance at a carboxy, amino, hydroxy, thiol etc.

The matrix may be attached to C, N or L (if present). From steric considerations it is believed that the most preferred attachment positions are in L and the least preferred positions are in N. The terminal position in the C-moiety is considered to be of intermediate or high preference. The most clear-cut positive effects have been accomplished for coupling

through a terminal position in C if it is a peptide. See the experimental part.

In order to have optimised ligands for affinity removal of a protein kinase from a liquid, bifunctional inhibitor analogues selected in accordance with the information presented above will have to be tested and if necessary modified. Testing may be carried out as described in the experimental part.

The matrix and the attachment of the ligand thereto.

In the preferred modes of the invention, the ligand is attached to a base matrix that is insoluble in the aqueous media used. Such matrices often are based on polymers that expose a hydrophilic surface to the aqueous media used, i.e. expose hydroxy (-OH), carboxy (-COOH), carboxamido (-$CONH_2$, possibly in N- substituted forms), amino (-NH_2, possibly in substituted form), oligo- or polyethylenoxy groups, on external and, if present, also on internal surfaces. Typically the matrices are of the same kind as those normally used as chromatographic matrices. The polymers may, for instance, be based on polysaccharides, such as dextran, starch, cellulose, pullulan, agarose etc, which, if necessary, have been cross-linked, for instance with bisepoxides, epihalohydrins, 1,2,3-trihalo substituted lower hydrocarbons, to provide a suitable porosity and rigidity. The matrices may also be based on synthetic polymers, such as polyvinyl alcohol, poly hydroxyalkyl acrylates, poly hydroxyalkyl methacrylates, poly acrylamides, polymethacrylamides etc. In case of hydrophobic polymers, such as those based on divinyl and monovinyl substituted benzenes, the surfaces of the matrices are often hydrophilized to expose hydrophilic groups as defined above to a surrounding aqueous liquid.

The matrices may also be of inorganic nature, e.g. silica, zirkonium oxide etc.

Physically the insoluble matrices may be in the form of porous monoliths or in beaded/particle form that can be porous

or non-porous. Matrices in beaded/particle form can be used as a packed bed or in a suspended form. Suspended forms include so called classified expanded beds and pure suspensions in which the particles/beads are moving round completely at random. In case of monoliths, packed bed and classified expanded beds, the separation procedure may be classified as a normal chromatography with a concentration gradient of adsorbed molecules being established along the flow direction. In case of pure suspension the separation procedure will be in the batch wise mode.

The ligand may be attached to a matrice by the use of conventional coupling techniques utilising, e.g. amino and/or carboxy groups present in the ligand. In case a cysteine residue is present it may be utilized as well (thioether or disulfide attachments). Bisepoxides, epichlorohydrin, CNBr, N-hydroxysuccinimide (NHS) etc are typical coupling reagents.

Between the base matrix and the ligand there is often introduced a spacer that will improve the availability of the ligand and facilitate the chemical coupling of the ligand to the matrix. Generally the spacer provides a hydrocarbon chain that has a length between 1-50 atoms. The hydrocarbon chain may be straight, branched or cyclic. The chain may be optionally interrupted by one or more ether oxygen or amino nitrogen atoms and/or optionally substituted with one or more hydroxy, lower alkoxy, or amino group ($-NH_2/NH_3^+$, where each hydrogen may be replaced with a lower alkyl or a lower acyl group). By lower alkyl or acyl group is primarily intended C_1-C_{10} alkyls/acyls. The spacer group may also, depending to immobilization methodology, comprise ester, amido, thioether, etc groups that have the sufficient hydrolytic stability.

Each spacer may carry one or more ligands ($C-L_n-N$) such that there may be an organic link chain of one or many atoms between each ligand.

It can be envisaged that the ligand may also be attached to the matrix by non-covalent bonding, such as physical

adsorption or biospecific adsorption. For the latter type of binding the biotin-strepavidin system may be utilized.

As a potential alternative the ligand may be in soluble form that subsequent to binding a protein kinase is insolubilized. This may be accomplished, for instance, by utiizing a ligand-biotin conjugate and contacting the formed affinity complex between the protein kinase and the soluble ligand-biotin conjugate with a strepavidin-matrix.

Procedural steps

During the adsorption step the conditions are selected so as to promote binding between the ligand and the protein kinase without irreversibly denaturing the kinase. pH is typically selected between 4-10, with preference for 6-9, the ionic strength in the interval corresponding to 0-1 M NaCl, and the temperature in the interval 0-40°C, with preference for 4-37°C. The exact values will depend on the particular protein kinase that is to be removed/purified and on the bifunctional inhibitor ligand attached to the matrix. After adsorption the protein kinase bound to the ligand may be further worked up, for instance by first desorbing the bound protein kinase and subsequently subjecting it to further adsorption steps, for instance on an ion exchanger. Suitably desorption conditions may include change of pH, ionic strength, temperature and or addition of compounds interfering with binding. Appropriate desorbing compounds may be selected from free ligands C-$(L)_n$-N and other soluble compounds exhibiting structures as defined for C and N or suitable part structures thereof competitively inhibiting binding between the kinase and the ligand. Depending on the protein kinase to be desorbed, nucleotide phosphates, such as adenosine-5'- (ATP and analogues thereof, ADP, AMP), adenosine and guanosine-5'-phosphates can be used typically in combination with magnesium(2+) ions. Basic or acidic amino acids and peptides may also be used depending, for instance, on the kind of kinase kinase. For certain

protein kinases it may also be possible to use polyions, for instance polyanions such as heparin. In certain cases the protein kinase may be also eluted with salt.

The main rule for both affinity binding and desorption is not to adapt the conditions such that the protein kinase concerned becomes irreversibly denatured.

There may be other adsorption steps preceding the binding of the protein kinase to the ligand. Such steps may for instance be based on ion exchange or on immune ligands.

The sample/liquid containing the protein kinase that is to be removed/purified

The protein kinase that is to be removed and/or purified typically exists in mixture with other proteins and/or biomolecules. The sample/liquid may be

(a) a blood preparation (such as plasma and serum),

(b) a fermentation liquid obtained from cultured host cells that have been transformed to express a protein kinase,

(c) a working up preparation derived from any one of the liquids mentioned in (a) and (b),

(d) various preparations of living tissue, such as extracts, homogenisates etc from animal, bacterial, yeast or plant origin.

Typically the sample/liquid is aqueous, such as a water solution.

E X P E R I M E N T A L P A R T

Abbreviations: Boc = t-butoxycarbonyl; Bop = benzotriazolyloxy tris(dimethylamino)phosphonium hexaflurophosphate; Fmoc = 9-fluorenylmethoxycarbonyl; Hobt = N-hydroxybenzotriazole; HPLC = high performance liquid chromatography; Pmc = 2,2,5,7,8-pentamethyl chroman-6-sulphonyl

EXAMPLE 1. Sunthesis of bifunctional inhibitor analogue ligand AdoC-Aoc-Arg_4-Lys

Wang-type peptide synthesis resin, Fmoc-Lys(Boc)-Resin (200 mg, 0.6 mmol/g; Advanced ChemTech) was swollen in dimethylformamide (DMF). Fmoc-protection was removed by treatment of the resin with a solution of 20% piperidine in DMF. The following arginines were attached to the resin in the form of Fmoc-Arg(Pmc) with Bop/Hobt activation. After removal of the N-terminal Fmoc-protection the following 8-Fmoc-aminooctanoic acid and 2', 3'-isopropylideneadenosine-5'-carboxylic acid were attached to the peptide chain with Bop/Hobt activation. Cleavage of the ligand from the resin and removal of the protection groups was achieved by treatment of the resin with a cocktail containing triisopropylsilane (100 µl), ethanedithiol (100 µl), water (100 µl) and TFA (2 ml, trifluoro acetic acid) for 2 hours. AdoC-Aoc-Arg_4-Lys was purified by an acetonitrile-water (0.1% TFA) gradient on a preparative C18 HPLC column. Lyophilization of the solution gave the pure ligand in the form of trifluoroacetate salt.

Coupling of the ligand to Sepharose. 0.4 g of freeze-dried Epoxy-activated Sepharose 6B (APB) was washed with 50 mL of water. The ligand AdoC-Aoc-Arg_4-Lys (10 mg) was dissolved in 2.0 mL of 0.1 M carbonate buffer (pH=10.5) and added to the gel suspension. After the coupling of the ligand on a shaker (60 hours at room temperature) remaining epoxy groups were blocked with 2-mercaptoethanol in the same carbonate buffer. The gel was washed with carbonate (0.1 M, pH 9.5) and acetate (0.1 M, pH 4.5) buffers and water.

EXAMPLE 2. Chromatography.

0.5 ml affinity column was equilibrated with 20 mM Tris-HCl, pH 7.5. One volume of cell homogenate of *E.coli* containing the overexpressed catalytic subunit of protein kinase A and 45

mg/ml of total protein was diluted with two volumes of equilibration buffer containing NaCl to final salt concentration of 200 mM. After loading of 200 µl of the diluted *E.coli* cell homogenate the column was washed with 6-10 volumes of equilibration buffer containing 200 mM NaCl. The protein kinase A catalytic subunit was then eluted with 1mM ATP, 20 mM $MgCl_2$giving more than 95% pure protein.

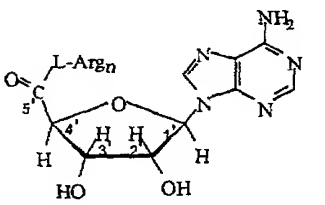
As an alternative protocol for elution the stepwise elution with L-arginine was used. The protein kinase A started to elute at 100 mM L-Arg while the most material came off at 150 and 200 mM. The purified protein can be considered at least 95% pure.

For regeneration of the column a washing step with 1.2 M NaCl was included.

EXAMPLE 3. Bifunctional inhibitor analogues with N = adenosine-5'-carboxylic acid (AdoC), C = Arg_6, Arg_4 or Arg_2, and various L.

Table 1

The general formula of protein kinase inhibitors in table 1.



No	Inhibitor[a)]	Linker structure
I	AdoC(β-Ala)$_2$AlaArg$_6$	[NHCH$_2$CH$_2$C(O)]$_2$NHCH(CH$_3$)C(O)
II	AdoC(Dpr)$_2$AlaArg$_6$	[NHCH$_2$CH(NH$_3^+$)C(O)]$_2$NHCH(CH$_3$)C(O)
III	AdoC(β-Asp)$_2$AlaArg$_6$	[NHCH$_2$CH(COO$^-$)C(O)]$_2$NHCH(CH$_3$)C(O)
IV	AdoCGlyArg$_6$	NHCH$_2$ C(O)

V	AdoC(β-Ala)Arg_6	$NH(CH_2)_2C(O)$
VI	AdoC(GABA)Arg_6	$NH(CH_2)_3C(O)$
VII	AdoC(Ahx)Arg_6	$NH(CH_2)_5C(O)$
VIII	AdoC(Aoc)Arg_6	$NH(CH_2)_7C(O)$
IX	AdoC(Aun)Arg_6	$NH(CH_2)_{10}C(O)$
X	AdoC(Ahx)Arg_4	$NH(CH_2)_5C(O)$
XI	AdoC(Ahx)Arg_2	$NH(CH_2)_5C(O)$
XII	Arg_6	-
XIII	Ac(Ahx)Arg_6	-
XIV	Ado	-

[a] The peptide and the linker were assembled by conventional Fmoc-peptide chemistry and 2'-, 3'-isopropylideneadenosine-5'-carboxylic acid was attached by coupling with BOP/Hobt activation (Uri et al., Bioorg. Med. Chem. 2 (1994) 1099-; and Pehk et al Bioorg. Med. Chem. Lett. 7 (1997) 2159-). The compounds were purified by an acetonitrile gradient with 0.1% TFA on a preparative C18 HPLC column. The products were analysed on a cation exchange column Mono S HR 5/5 (Pharmacia Biotech) and on a MALDI TOF mass-spectrometer Kratos Kompact IV. The experimental molecular mass values agreed well with the calculated values.

Among the arginine-peptide containing compounds (Table 1) several potent inhibitors of PKA, PKC and Ca2+-dependent protein kinase 1 (CDPK-1) (Muszynska, et al., Biochem. Mol. Biol. Int. 30 (1993) 849-; and Loog et al., Bioorgan. Med. Chem. Lett. 9 (1999) 1447-1452) were found, while no significant inhibition of protein kinases CK1 and CK2 was observed (Table 2). This result was not surprising, as the substrate specificity of PKA and PKC (Kennelly et al., J. Biol. Chem. 266 (1991) 15555-).as well as of CDPK-1 (Muszynska, et al., Biochem. Mol. Biol. Int. 30 (1993) 849-; and Loog et a., manuscript submitted) is clearly governed by positively charged amino acids, while protein kinases CK1 and CK2 phosphorylate only negatively charged peptide sequences (Pinna et al., Biochim. Biophys. Acta 1314 (1996) 191-). Thus, the arginine-peptides were effective to direct the inhibitors

towards PKA, PKC and CDPK-1. Similarly, it can be assumed that a negatively charged "anchoring" peptide fragments will direct the inhibitors into the active centre of protein kinases CK1 and CK2.

Table 2

Interaction of protein kinase A (PKA), protein kinase Cβ (PKC), Ca^{2+}-dependent protein kinase from maize seedlings (CDPK-1), protein kinase CK1 and protein kinase CK2 with adenosine-5'-carboxylic acid derivatives as protein kinase inhibitors.

Inhibitor	IC_{50} (μM) [e]				
	PKA [a]	PKC [b]	CDPK-1 [c]	CK1 [d]	CK2 [d]
I	4.3±0.3	2.8±0.3	16±4	>100	>30
II	2.6±0.3	1.2±0.1	31±3	5.6±0.4	17±3
III	11.1±0.6	27±2	>50	>30	>30
IV	4.0±0.2	10±1	67±7	>30	>30
V	1.8±0.1	3.0±0.2	38±7	>30	n. i. [f]
VI	1.3±0.2	1.5±0.1	19±2	>30	>30
VII	0.12±0.02	0.27±0.01	1.2±0.2	>30	>30
VIII	0.24±0.02	0.14±0.01	3.2±0.1	>30	>30
IX	0.33±0.02	0.32±0.02	4.9±0.5	>30	n. i. [f]
X	1.2±0.1	-	52±8	-	-
XI	13.8±1.0	6.9±0.3	35±3	-	-

[a] The catalytic subunit of murine PKA was overexpressed and purified from *E.coli* strain BL21 (DE3) (Yonemoto et al., Methods Enzymol. 200 (1991) 581-). The expression vector

construct was kindly provided by Dr. S.S. Taylor (California, La Jolla) (Loog et al., Bioorg. Med. Chem. Lett 9 (1999) 1447-1452 (table 2, footnote a). PKA activity was assayed at 30°C in 60 mM Tris/HCl, pH 7.5, containing 3 mM 2-[N-morpholino] ethanesulfonic acid, 0.002 % Triton X-100, 0.3 mM EDTA, 0.2 mg/mL bovine serum albumin, 100 μM of Kemptide (Loog et al., Bioorg. Med. Chem. Lett 9 (1999) 1447-1452 (table 2, footnote a), 5 mM $MgCl_2$ and 30 μM [γ-^{32}P] ATP.

[b] Protein kinase C β isoform (PKC) was isolated from pig spleen (Parker et al., EMBO J. 3 (1984) 953-; and Ferrari et al., FEBS Lett. 184 (1985) 72-). PKC activity was assayed at 30°C in 50 mM Tris/HCl, pH 7.5, containing 0.002 % Triton X-100, 0.75 mM calcium acetate, 60 μg/mL phosphatidylserine, 1 μg/mL diolein, 0.3 mM EDTA, 0.2 mg/mL bovine serum albumin, 15 μM of substrate peptide (Toomik et al., Biochem. J. 322 (1997) 455- and Loog et al., Bioorg. Med. Chem. Lett 9 (1999) 1447-1452 (table 2, footnote a) , 5 mM $MgCl_2$ and 30 μM [γ-^{32}P] ATP.

[c] Ca^{2+}-dependent protein kinase (CDPK-1) was isolated from maize seedlings (Muszynska et al., Biochem. Mol. Biol. Int. 30 (1993) 849-; and Loog et al., manuscript submitted). The activity of CDPK-1 was assayed at 30°C in 45 mM Tris/HCl, pH 7.5, 0.005% Triton X-100, 0.7 mM $CaCl_2$, 0.4 mM EDTA, 0.4 mg/mL bovine serum albumin, 50 μM substrate peptide (number V in table 2 of Loog et al., Eur. J. Biochem. 267 (2000) 337-343), 5 mM $MgCl_2$, 30 μM [γ-^{32}P]ATP and 0.2-0.4 μg of the protein kinase pool.

[d] Protein kinases CK1 and CK2 were purified from rat liver as described by (Meggio et al., J. Biol. Chem. 256 (1981) 11958-). The activity was assayed at 30°C in 60 mM Tris/HCl, pH 7.5, 1 mg/mL casein, 100 mM NaCl, 2.5% glycerol, 5 mM $MgCl_2$, CK1 or CK2 and 30 μM [γ-^{32}P] ATP.

[e] The reactions were monitored by transferring 25 μL aliquotes onto 2 × 2 cm pieces of phosphocellulose paper, thereafter washed with 75 mM phosphoric acid five times and the bound radioactivity was measured. The IC_{50} values were obtained from the initial activity vs inhibitor concentration plots.

[f] n. i. means no inhibition at 100 μM concentration of ligand .

The tested bifunctional inhibitors contained structural elements from both substrates of protein kinases (ATP and peptide). If tested separately, adenosine and peptide fragments alone had a much weaker inhibition capacity compared to the bifunctional inhibitors (Table 3).

Table 3

Compound/Inhibitor	PKA*	PKC*
	K_i(nM)**	K_i(nM)**
Arg_6	29493	7440
Ac(amino-hexanoic acid)Arg_6	27880	8823
Adenosine	43090	43200
AdoC(amino-hexanoic acid)Arg_6	40.1	6.32

* Protein Kinase A and C, respectively

** Inhibition constant (affinity constant for binding of inhibitor to enzyme.

However, their combination *via* a linker group L increased the effectiveness of their interaction with the enzyme and yielded potent inhibitors of PKA, PKC and CDPK-1. The ionic status of L was varied using β-alanine, β-aspartic and 2,3-diaminopropionic acids for coupling the peptide and nucleoside parts, yielding differently charged linkers of the same backbone structure (compounds ***I***, ***II***, and ***III*** in Table 1). The IC_{50} values for these compounds with non-ionic and cationic linkers were similar, while the introduction of β-aspartic acid residues slightly decreased the potency of the inhibitors. This means that the ionic charges of the linker did not participate in the electrostatic stabilisation of the enzyme-inhibitor complex. As the linker part of the inhibitor presumably resides in the same region of the active site as the phosphate backbone of ATP in the reaction complex, these results were in agreement with the mutually close values of the affinities of ATP (K_d = 25 μM 21. (Lew et al., J. Biol. Chem. 272 (1997) 1507-)) and adenine (K_d = 30 μM (Narayana et al., Biochemistry 36 (1997) 4438-)) to PKA. Thus the charged phosphate moiety (as well as ribose) seems to have no clear contribution to the observed binding affinity. This could explain the failure of our attempts to increase the inhibitory

potency of the bisubstrate-analog inhibitors by introducing charges into the linker region.

Secondly, the flexibility of the linker was varied by replacing the conformationally restricted peptide-like groups by aliphatic chains of various length (compounds ***IV*** to ***IX*** in Table 1). All these compounds were effective inhibitors of PKA, PKC and CDPK-1, while maximal inhibitory effect was clearly depending on the linker length and these dependencies were somewhat different for the investigated enzymes.

Further, the potency of the inhibitors was clearly dependent on the length of the peptide fragment attached, pointing to a significant role of this part of the molecules in the specific interaction with the enzyme. Therefore it may be assumed that a variation of the peptide fragment structure, taking into account the details of the differential specificity of various protein kinases, may be a promising approach for a more selective targeting of these inhibitors.

The clear structural requirements for inhibition point to the importance of the compatibility of the inhibitor molecule with the binding sites for ATP and the peptide. These sites are located on different lobes of the catalytic subunit of PKA, where the upper lobe has the adenosine binding pocket and the lower lobe accommodates the peptide/protein binding site (Knighton et al., Science 253 (1991) 407-). These lobes are believed to move towards each other and bring the substrates together during the catalytic act. On the other hand, the protein conformation should be rather opened to allow the substrate binding. Therefore the spatially separated location of these binding sites may well be the main structural factor determining the length and the chemical properties of the linker group.

C L A I M S

1. A method for removal of protein kinase from a liquid containing the protein kinase by contacting the liquid with a carrier bound affinity ligand for the kinase, **characterized** in that the ligand is a bifunctional inhibitor for the kinase.

2. The method of claim 1, **characterized** in that the bifunctional inhibitor comprises the structure

$$C-(L)_n-N$$

where

(a) C contains a structure inhibiting binding of the peptide/protein substrate to the the protein kinase,

(b) L is an organic linker,

(c) n is an integer 0 or 1, and

(d) N is aninhibitor competitively inhibiting binding of the nucleoside triphosphate (NTP) to the protein kinase.

3. The method of any one of claims 1-2, **characterized** in that C is a peptide substrate consensus sequence or a pseudosubstrate consensus sequence.

4. The method of claim 3, **characterized** in that C comprises at least one basic amino acid residue, preferably selected from arginine, lysine and hydroxylysine, when the protein kinase has basic substrate specificity determinants.

5. The method of claim 3, **characterized** in that C comprises one acidic amino acids, preferably selected from aspartic acid and glutamic acid, when the protein kinase has acidic substrate specificity determinants.

6. The method of any one of claims 3-5, **characterized** in that C comprises at least one hydrophobic amino acid residue, preferably selected among leucine, isoleucine, phenylalanine.

7. The method of any one of claims 3-6, **characterized** in that C comprises at least two amino acid amino acids and/or at least two basic amino acids, with preference for these amino acid residues being in sequence.

8. The method of any one of claims 3-7, **characterized** in that C comprises at least two arginine residues and/or at least two aspartic acid residues.

9. The method of any one of claims 2-8, **characterized** in that N is a structure comprising a nucleotide structure, preferably adenosine or guanosine with the protein kinase being capable of phosphorylate with ATP and GTP, respectively.

10. The method of claim 9, **characterized** in that the nucleotide structure is covalently bound to L utilizing its 5'-carbon.

11. The method of claim 10, **characterized** in that the 5'-carbon is in the form of a derivatized carboxylic acid.

12. The method of any one of claims 2-11, **characterized** in that L is a peptide chain.

13. The method of anyone of claims 2-12, **characterized** in that L is a peptide chain composed of non-α- and/or non-L amino acids.

14. The method of any one of claims 1-15, **characterized** in that the carrier is insoluble in the liquid.

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (12-97)
Approved for use through 9/30/00. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

[X] Declaration Submitted with Initial Filing **OR** [ ] Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	PL-9515
First Named Inventor	M. Loog
COMPLETE IF KNOWN	
Application Number	To be assigned
Filing Date	29-Oct-2001
Group Art Unit	To be assigned
Examiner Name	To be assigned

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Method for the Purification of Protein Kinase by Affinity Chromatography

(Title of the Invention)

the specification of which
[X] is attached hereto
OR
[ ] was filed on (MM/DD/YYYY) [ ] as United States Application Number or PCT International Application Number [ ] and was amended on (MM/DD/YYYY) [ ] (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
9901807-9	Sweden	05/17/1999	[ ]	[ ]	[X]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

[ ] Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

Burden Hour Statement: This form is estimated to take 0.4 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (12-97)
Approved for use through 9/30/00. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
PCT/EP00/04104	05/18/2000	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: ☒ Customer Number 22840 → *Place Customer Number Bar Code Label here*

OR

☐ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☒ Customer Number or Bar Code Label 22840 *OR* ☐ Correspondence address below

Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
Mart	Loog

Inventor's Signature						Date	
Residence: City		State		Country	EE	Citizenship	EE
Post Office Address	Rua 11-12						
Post Office Address	Tartu Estonia EE51010						
City		State		ZIP		Country	

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

Please type a plus sign (+) inside this box → [+]

PTO/SB/02A (3-97)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 1

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Asko			Uri				
Inventor's Signature						**Date**	
Residence: City		**State**		**Country**	EE	**Citizenship**	EE
Post Office Address	Paeva St. 23-2						
Post Office Address	Tartu Estonia EE50103						
City		**State**		**ZIP**		**Country**	

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Jaak			Jarv				
Inventor's Signature						**Date**	
Residence: City		**State**		**Country**	EE	**Citizenship**	EE
Post Office Address	Wiiralti St.m 31-4						
Post Office Address	Tartu Estonia EE51010						
City		**State**		**ZIP**		**Country**	

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])			Family Name or Surname				
Pia			Ek				
Inventor's Signature						**Date**	
Residence: City		**State**		**Country**	SE	**Citizenship**	SE
Post Office Address	Nyhagen, S-740 30						
Post Office Address	Bjorklinge Sweden						
City		**State**		**ZIP**		**Country**	

Burden Hour Statement: This form is estimated to take 0.4 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (12-97)
Approved for use through 9/30/00. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

☐ Declaration Submitted with Initial Filing OR ☒ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)

Attorney Docket Number	PL-9515
First Named Inventor	M. Loog
COMPLETE IF KNOWN	
Application Number	10 /018,021
Filing Date	29-Oct-2001
Group Art Unit	To be assigned
Examiner Name	To be assigned

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint invento r (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Method for the Purification of Protein Kinase by Affinity Chromatography

(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) 05/08/2000 as United States Application Number or PCT International Application Number PCT/EP00/04104 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inven tor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's cer tificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
9901807-9	Sweden	05/17/1999	☐	☐	☒
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

Please type a plus sign (+) inside this box → [+]

PTO/SB/01 (12-97)
Approved for use through 9/30/00. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*
PCT/EP00/04104	05/18/2000	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

☒ Customer Number 22840 → 22840 *Place Customer Number Bar Code Label here*

OR

☐ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☒ Customer Number or Bar Code Label 22840 *OR* ☐ Correspondence address below

Name					
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
1-00 Mart	Loog

Inventor's Signature						Date	31/01/2002
Residence: City		State		Country	EE	Citizenship	EE EEK
Post Office Address	Rua 11-12						
Post Office Address	Tartu Estonia EE51010						
City		State		ZIP		Country	

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.

Please type a plus sign (+) inside this box → [+]

PTO/SB/02A (3-97)
Approved for use through 9/30/98. OMB 0651-0032
Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 1

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])				Family Name or Surname			
2-00 Asko				Uri			
Inventor's Signature						**Date**	31/01/2002
Residence: City		**State**		**Country**	EE	**Citizenship**	EE EEX
Post Office Address	Paeva St. 23-2						
Post Office Address	Tartu Estonia EE50103						
City		**State**		**ZIP**		**Country**	

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])				Family Name or Surname			
3-00 Jaak				Jarv			
Inventor's Signature						**Date**	31/01/2002
Residence: City		**State**		**Country**	EE	**Citizenship**	EE EEX
Post Office Address	Wiiralti St.m 31-4						
Post Office Address	Tartu Estonia EE51010						
City		**State**		**ZIP**		**Country**	

Name of Additional Joint Inventor, if any:	☐ A petition has been filed for this unsigned inventor						
Given Name (first and middle [if any])				Family Name or Surname			
4-00 Pia				Ek			
Inventor's Signature						**Date**	12/2-2002
Residence: City		**State**		**Country**	SE	**Citizenship**	SE SEX
Post Office Address	Nyhagen, S-740 30						
Post Office Address	Bjorklinge Sweden						
City		**State**		**ZIP**		**Country**	

Burden Hour Statement: This form is estimated to take 0.4 hours to complete. Time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, Patent and Trademark Office, Washington, DC 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Assistant Commissioner for Patents, Washington, DC 20231.